Evidence for Direct-acting Oxidative Genotoxicity by Reduction Products of Azo Dyes

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The intestinal flora forms a complex ecosystem that metabolizes dietary and endogenous nutrients under primarily anaerobic conditions. The ingestion of azo dyes has been proposed as one source of potential genotoxic agents. Many intestinal bacteria are able to reduce the azo bond (termed azofission), which liberates the substituted naphthol compounds. The standard Ames test has not demonstrated mutagenicity either by various common food colorings or by their reduced end products in *Salmonella typhimurium* strains TA98 and TA100. In contrast, genetic toxicity was demonstrated in the *Escherichia coli* differential kill assay and in *S. typhimurium* TA102 for the reduced dyes. The superoxide free radical was produced by the azo dyes only after reduction by the intestinal bacteria *Enterococcus faecalis* and *Bacteroides thetaiotaomicron*. — Environ Health Perspect 102(Suppl 6):119–122 (1994)

Key words: azo dyes, *Salmonella typhimurium* TA102, superoxide radicals, azo reduction, oxidative mutagens, genotoxicity

Introduction

Azo compounds are the most common synthetic colorings used in the food, pharmaceutical, and cosmetic industry. Also known as coal tar dyes, they contain an aromatic ring linked by an azo bond to a second naphthalene or benzene ring. Coloring matter entering the intestinal tract is subjected to the action of acid, digestive enzymes, and microflora. Azo compounds may reach the intestine directly after oral ingestion or through the bile after parenteral administration. They are reduced by azo reductases from intestinal bacteria and, to a lesser extent, by enzymes of the cytosolic and microsomal fractions of the liver. The first catabolic step in the reduction of azo dyes, which is accompanied by a decrease in the visible light absorbance and then decoloration of the dye, is the reduction of the azo bond to produce aromatic amines (Figure 1). Aromatic amines, some of which are known carcinogens, have been found in the urine of dyestuff workers and test animals following administration of azo dyes (*1*).

Although a number of commonly used dyes are not mutagenic in *Salmonella typhimurium* strains TA98 and TA100 even after azoreduction, the production of reactive oxygen species from *o*-hydroxy aromatic amine products has been suggested (*2,3*). This article reports the generation of superoxide anions from reduced azo dyes and aminonaphthols and the genetic toxicity of these products in *Escherichia coli* and a *Salmonella* strain (TA102), which is sensitive to oxidants.

Materials and Methods

Bacterial Strains

The organisms studied were obtained from The Nottingham Trent University Culture Collection (England), except *S. typhimurium* strains, which were obtained from B. Ames, University of California, Berkeley.

Enterococcus faecalis was grown in tryptone soya broth, and *Bacteroides thetaiotaomicron* was grown anaerobically in Schaedlers broth; both were incubated at 37°C for 24 hr. Cells were harvested by centrifugation at 7890*g* for 10 min and anaerobically washed once with 0.5 M potassium phosphate buffer, pH 7.4. The bacterial pellets were resuspended to one-twentieth of their previous volume in phosphate buffer and immediately used for azo reduction.

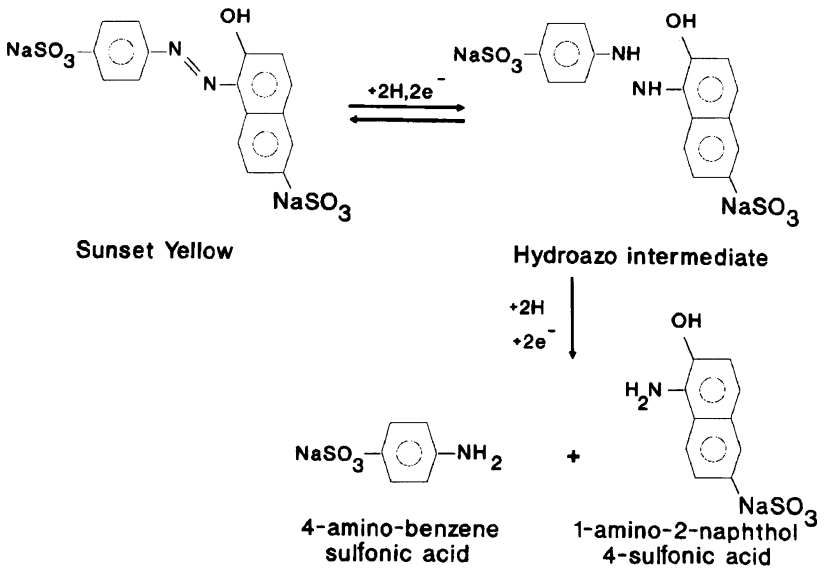

Figure 1. Possible reduction mechanism for sunset yellow.

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Materials

Sunset yellow (Colour Index [C.I.] 15985) and carmoisine (C.I. 14720) were obtained from Gurr, BDH Chemicals Ltd (Poole, Dorset, UK). Amaranth (C.I. 16185) and all other chemicals used were obtained from Sigma Chemical Co. (Poole, Dorset, UK). It should be noted that purity of the azo dyes was 85%. The electron carriers used were flavin mononucleotide (FMN), riboflavin (RF), and flavin adenine dinucleotide (FAD).

Bacterial Reduction of Azo Dyes

The standard assay mix consisted of 4.2 ml degassed potassium phosphate buffer (50 mM, pH 7.4), 0.4 ml washed cell suspension with or without glucose (0.2 ml, 10% wt/vol) and electron carriers in narrow assay tubes (18 × 142 mm). The various electron carriers were prepared anaerobically at 0.025 mM. The reaction mix was incubated at 37°C prior to starting the reaction by the addition of 0.1 ml of the appropriate azo dye (10 mM). Decoloration rate was determined spectrophotometrically at the wavelength of the maximal absorbance of the azo dye.

Chemical Reduction Assay

Dyes were reduced either with sodium dithionite according to the method of Zbaida and Levine (*4*), or with stannous chloride as described by Gasparic (*5*).

Detection of Superoxide Anion Radical

Superoxide anion (O_2^-) production was detected by the reduction of nitroblue tetrazolium (NBT) and its inhibition by superoxide dismutase (SOD), according to the method of Oberley et al. (*6*). The sample (100 µl) to be tested was added to 0.8 ml potassium phosphate buffer (50 mM, pH 7.8) containing 0.056 mM NBT, 0.1 mM EDTA, 0.1 mM xanthine, 0.06% wt/vol Triton X100, and 0.33 mg/ml gelatin, ± 100 µl SOD. Immediately after mixing, the absorbance at 560 nm was measured with respect to time. SOD activity was assayed using a xanthine/xanthine oxidase technique (*6*). SOD was inactivated by boiling for 15 min.

Differential Killing Assay

The *E. coli* differential cytotoxicity assay was used to determine the genotoxic activity of the azo dyes according to the method described by Tweats et al. (*7*). The tester strains were *E. coli* WP2, (repair proficient) and *E. coli* CM871 (repair deficient). *E. coli* CM871 is a uvrA recA lexA triple mutant that combines extreme repair deficiency with near wild-type growth. Compounds were accepted as genotoxic if survival of the repair deficient strain was at least 4-fold lower than survival of the repair proficient strain.

All compounds were tested for genotoxicity with and without prior incubation with either *E. faecalis* or *B. thetaiotaomicron* cell suspensions. Incubation was for 1 hr with *E. faecalis* or overnight with *B. thetaiotaomicron*. Final concentrations of agents used were azo dyes, 5 mM; 1-aminonaphthol HCl, 0.1 mM; 1-aminonaphthol-4-sulfonic acid, 10 mM; 4-aminonaphthalene HCl, 1 mM; and 4-aminonaphthalene-sulfonic acid sodium salt, 5 mM.

Genotoxic activity was assessed after removing cells by centrifugation in a microfuge (8000*g*, 5 min), followed by filtration (0.2 µm Dynaguard filters, New Brunswick). An aliquot (100 µl) of the filtrate was added to the diluted *E. coli* tester strain (200 µl), and the genotoxic activity was determined. Inhibition by UV was used as a positive control for CM871.

The Rapid Automated Bacterial Impedance Technique (RABIT), (Don Whitley Scientific Ltd, Shipley, UK) and plate counts were used to measure microbial survival (*8*).

Salmonella Mutagenicity Test

The protocol used was essentially that of Ames et al. (*9*). The strains of *S. typhimurium* used were TA98, TA100, and TA102. Liver postmitochondrial supernatant was not incorporated in this test. Bleomycin was used as a positive control for TA102 (*9*).

Results

The reduction of the three dyes by *E. faecalis* and *B. thetaiotaomicron* is summarized in Table 1. Azo reductase activity was stimulated by the addition of extracellular electron acceptors such as FMN, FAD, and RF. The presence of glucose on reduction of azo dyes was inhibitory for *B. thetaiotaomicron*, but not for *E. faecalis*. This may be because of the different electron transport systems of these organisms.

After incubation with *E. faecalis*, the cleavage products of sunset yellow and amaranth were found to be genotoxic in the bacterial differential assay (Figure 2). Carmoisine was marginally genotoxic after azo reduction, but survival of *E. coli* CM871 was not consistently 4-fold less than survival of *E. coli* WP2. Similar results were obtained on incubation with *B. thetaiotaomicron*. Unreduced dyes were not genotoxic in this assay. Of the four coupling components tested, 1-amino-2-naphthol HCl and 1-amino-2-naphthol-4-sulfonate are considered genotoxic.

Mutagenicity studies on the same samples using the standard Ames test with *S. typhimurium* TA98 and TA100 showed no mutagenicity. When compounds were retested using *S. typhimurium* TA102, reduced amaranth and reduced sunset yellow gave a doubling of the spontaneous reversion rate (Figure 3). This strain detects a variety of oxidants as mutagens that are not detected by strains TA98 or TA100 (*10*).

Generation of O_2^- was observed for reduced dyes and some aminonaphthols but not for unreduced dyes (Figures 4,5). The increase in absorbance at 540 nm (NBT assay) was inhibited by SOD, while inactivated SOD showed no inhibition. Table 2 summarizes the rates of O_2^- production from unreduced and reduced azo dyes and their coupling components. Results were further confirmed by an ability to reduce cytochrome *c* (data not shown). Negligible

Table 1. Cofactor requirements for azoreductase activity.[a]

Compound	Cofactor	Rate of decoloration, µmole/min/mg cell dry wt by *E. faecalis*[b]	by *B. thetaiotaomicron*[c]
Amaranth	–	NQ	NQ
Amaranth	Gl	40.7 ± 2.4	NQ
Amaranth	FMN	NQ	7.2 ± 1.24
Amaranth	Gl + FMN	113.5 ± 7.04	3.9 ± 0.69
Amaranth	Gl + FAD	83.6 ± 3.5	5.5 ± 0.36
Amaranth	Gl + RF	75.6 ± 5.36	4.6 ± 0.17
Sunset yellow	–	NQ	NQ
Sunset yellow	Gl	79.0 ± 1.5	NQ
Sunset yellow	FMN	3.9 ± 0.17	7.5 ± 0.21
Sunset yellow	Gl + FMN	132.5 ± 4.11	5.41 ± 0.25
Sunset yellow	Gl + FAD	93.5 ± 1.98	6.3 ± 0.25
Sunset yellow	Gl + RF	78.1 ± 3.6	4.6 ± 0.48

Abbreviations: NQ, nonquantifiable (rate less than 1 nm dye reduced per minute per milligram cell dry weight); Gl, glucose; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide. [a]Mean ± SD of three independent exponents. [b]Cell dry weight = 0.76 mg/ml. [c]Cell dry weight = 0.51 mg/ml.

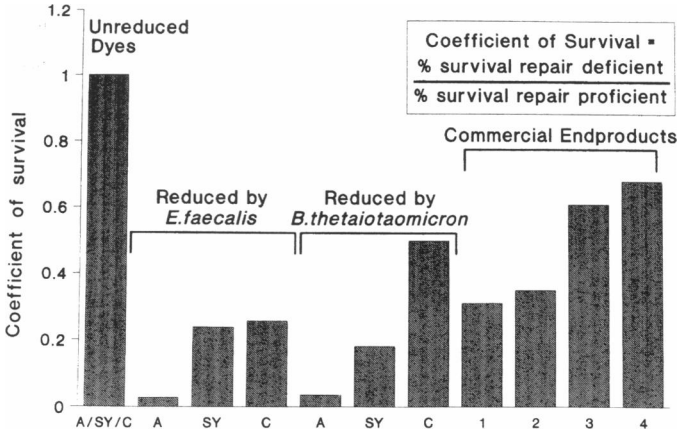

Figure 2. Bacterial genotoxicity of azo dyes using bacterial differential assay. A, amaranth; SY, sunset yellow; C, carmoisine; 1) 1-amino-2-naphthol hydrogen chloride; 2) 1-amino-2-naphthol-4-sulfonic acid; 3) 4-aminonaphthalene HCl; 4) 4-aminonaphthalene sulfonic acid, sodium salt.

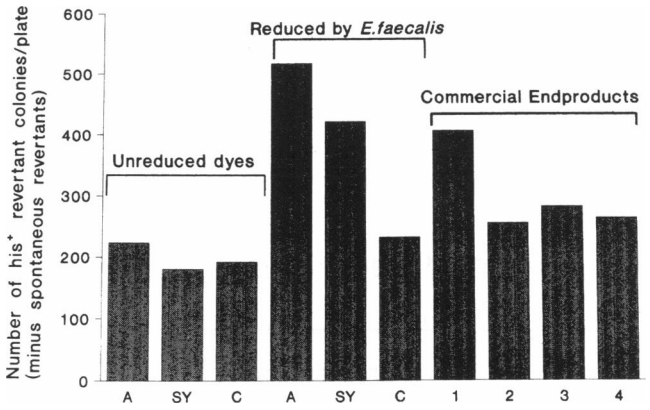

Figure 3. Bacterial mutagenicity of azo dyes, Ames test, and *S. typhimurium* TA102. A, Amaranth; SY, sunset yellow; C, carmoisine; 1) 1-amino-2-naphthol hydrogen chloride; 2) 1-amino-2-naphthol-4-sulfonic acid; 3) 4-aminonaphthalene HCl 4) 4-aminonaphthalene sulfonic acid, sodium salt. The mean number of viable bacteria and plate for spontaneous and control (1 mg/ml) incubations were 140 and 1050.

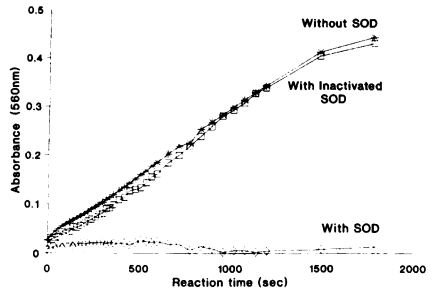

Figure 4. Superoxide production from sunset yellow reduced by *E. faecalis*.

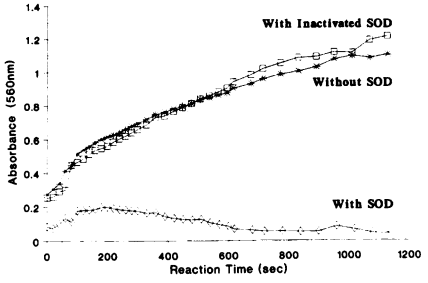

Figure 5. Superoxide production from sunset yellow reduced by stannous chloride.

O_2^- was produced by 4-aminonaphthalene and its sodium salt.

Discussion

It has been demonstrated that there are no cytochromes present in cell-free extracts of *E. faecalis* and that the transfer of electrons from reduced pyridine nucleotides to electron acceptors was mediated by a flavoprotein (*11*).

In view of their charged nature, it is uncertain whether FAD and FMN could readily shuttle across bacterial cell walls and membranes under physiologic conditions. In addition, it is unlikely that highly charged sulfonated dyes could pass through the cell wall of these organisms. An electron shuttle could affect the reduction with the dyes remaining extracellular. It may also serve as a partial explanation for the oxygen sensitivity of these reduction reactions, reduced flavin having a greater affinity for oxygen than it has for the azo dye.

In this article, we have tested three azo dyes for genotoxicity following bacterial reduction of the dye. Both amaranth and sunset yellow, when reduced, induced cytotoxicity indicating DNA damage in repair-deficient *E. coli* in the absence of hepatic enzymes, but they failed to mutate *S. typhimurium* strains TA98 and TA100. In contrast, strain TA102, which detects oxidative mutagens, (*10,12*) was mutated by reduced amaranth and reduced sunset yellow.

The findings suggest that various azo dye products are genotoxic, not through *N*-hydroxylation and esterification, which is characteristic of many aromatic amines (*2,13*), but rather through a mechanism involving oxygen radicals (*3,14*). In further support, the production of O_2^- radical from reduced azo dyes was detected by the

Table 2. Generation of O_2^- naphthalamines.[a]

Compound	Rate of O_2^- production, μmole compound/min
Xanthine	0.014 ± 0.0021
Amaranth	0
Carmoisine	0
Sunset yellow	0
Amaranth[b]	0.023 ± 0.0017
Carmoisine[b]	0.018 ± 0.0024
Sunset yellow[b]	0.026 ± 0.0011
Amaranth[c]	0.062 ± 0.0018
Carmoisine[c]	0.0198 ± 0.0034
Sunset yellow[c]	0.055 ± 0.0023
I-Amino-2-naphthol HCl	0.1065 ± 0.0045
4-Amino-2-naphthol sulfonic acid	0.022 ± 0.0026
4-Aminonaphthalene HCl	$1.336\times10^{-4} \pm 0.3\times10^{-5}$
4-Aminonaphthalene sulfonic acid, Na salt	$0.033\times10^{-4} \pm 0.4\times10^{-5}$

[a] Mean ± SD of three independent exponents. [b] Reduced by *E. faecalis*. [c] Reduced by $SnCl_2$.

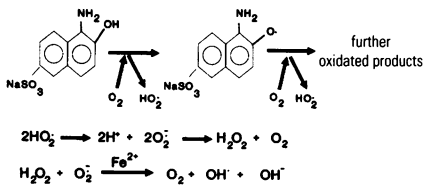

Figure 6. Possible mechanism for production of superoxide anions from reduced sunset yellow. Adapted from Nakayama (*3*).

reduction of nitrotetrazolium blue and confirmed using cytochrome *c*. The mechanism by which these species are formed is not clear, but they may be specific for *o*-hydroxy aromatic amines and involve the reactions postulated by Nakayama (*3*) and summarized in Figure 6. There may be a requirement for iron, as in Fenton chemistry, where O_2^- is converted via H_2O_2 to the highly reactive OH species that is known to damage DNA (*14*). All of the compounds shown to generate active oxygen species have a hydroxyl substituent *ortho* to the amino function. However, this does not explain the weakly mutagenic results for carmoisine. O_2^- was detected for 1-amino-2-naphthol HCl and 1-amino-2-naphthol sulfonate, while no active oxygen was detected for 4-aminonaphthalene HCl and its sodium salt.

We are currently studying the ability of *o*-hydroxy aromatic amines to produce deoxynucleotide oxidation in DNA. The identification of this type of DNA damage hitherto not detected by many conventional genotoxicity assays may have important implications regarding the continued use of azo dyes in foodstuffs.

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